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Synthesis of Ribavirin, Tecadenoson, and Cladribine by Enzymatic Transglycosylation

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Abstract: Despite the impressive progress in nucleoside chemistry to date, the synthesis of nucleoside analogues is still a challenge. Chemoenzymatic synthesis has been proven to overcome most of the constraints of conventional nucleoside chemistry. A purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNP) has been used herein to catalyze the synthesis of Ribavirin, Tecadenoson, and Cladribine, by a "one-pot, one-enzyme" transglycosylation, which is the transfer of the carbohydrate moiety from a nucleoside donor to a heterocyclic base. As the sugar donor, 7-methylguanosine iodide and its 2′-deoxy counterpart were synthesized and incubated either with the "purine-like" base or the modified purine of the three selected APIs. Good conversions (49–67%) were achieved in all cases under screening conditions. Following this synthetic scheme, 7-methylguanine arabinoside iodide was also prepared with the purpose to synthesize the antiviral Vidarabine by a novel approach. However, in this case, neither the phosphorolysis of the sugar donor, nor the transglycosylation reaction were observed. This study was enlarged to two other ribonucleosides structurally related to Ribavirin and Tecadenoson, namely, Acadesine, or AICAR, and 2-chloro-N^6-cyclopentyladenosine, or CCPA. Only the formation of CCPA was observed (52%). This study paves the way for the development of a new synthesis of the target APIs at a preparative scale. Furthermore, the screening herein reported contributes to the collection of new data about the specific substrate requirements of *Ah*PNP.

1. Introduction

Nucleoside analogues are well-established drugs in clinical practice; they are mainly used as anticancer and antiviral agents. However, the search for new therapeutically active nucleosides is still a vibrant research area, as witnessed by the approval of the pro-drug Sofosbuvir, marketed as Sovaldi® in 2013, used in the treatment of hepatitis C as an alternative to peginterferon-combined therapies (e.g., in association with Ribavirin and Daclatasvir, Ledipasvir or Simeprevir) [1].

Drug discovery stands alongside the set-up of new synthetic strategies aimed at circumventing the typical constraints of nucleoside chemistry (e.g., multi-step processes, protection/deprotection reactions, lack of selectivity, etc.) [2]. Chemoenzymatic synthesis has been proven to overcome most of these drawbacks. The main advantages of enzymatic methods include high catalytic efficiency, mild reaction conditions (and thus environmentally friendly and safer syntheses), high stereo- and regioselectivity, and fewer numbers of synthetic steps. However, a truly efficient synthesis of nucleoside analogues is often the result of a combination of chemical methods and biochemical transformations [3].

Purine nucleoside phosphorylases (PNPs, EC 2.4.2.1) catalyze the reversible cleavage of the glycosidic bond of purine nucleosides in the presence of inorganic orthophosphate as a co-substrate, to generate the conjugated nucleobase and α-D-pentofuranose-1-phosphate. If a second purine base is in the reaction medium, the formation of a new nucleoside can result by a regio- and stereoselective transglycosylation reaction [3].

Accumulated data about a PNP from *Aeromonas hydrophila* (*Ah*PNP) [4] have clearly shown that this enzyme can be successfully used in the synthesis of a wide range of nucleoside analogues, which are either routinely used as drugs (e.g., arabinosyladenine) [5,6] or can have promising pharmacological activities, such as some 6-substituted purine ribonucleosides [7,8]. This PNP has been shown to have a quite relaxed substrate tolerance toward the purine base, to recognize ribo- and 2′-deoxyribonucleosides as the sugar donor, and, although to a lesser extent, to accept D-arabinose-1-phosphate produced by the phosphorolysis of arabinosyluracil in a bi-enzymatic transglycosylation reaction [4–9].

Ribavirin (Virazole®) is considered the "gold-standard" in the treatment of hepatitis C in association with pegylated interferon-alpha (IFN-α) [10–13]. Tecadenoson and its congeners are selective A1 receptor agonists, which have been investigated for their use against arrhythmia and atrial fibrillation [14–16]. Cladribine (Litak®) has been approved for the treatment of symptomatic tricoleukaemia (hairy-cell leukemia). It can act both as a chemotherapy drug and an immunosuppressive agent. Clinical studies have also suggested its potential usefulness in the treatment of multiple sclerosis [17,18]. Chemical structures of Ribavirin (**1**), Tecadenoson (**2**), and Cladribine (**3**) are reported in Figure 1.
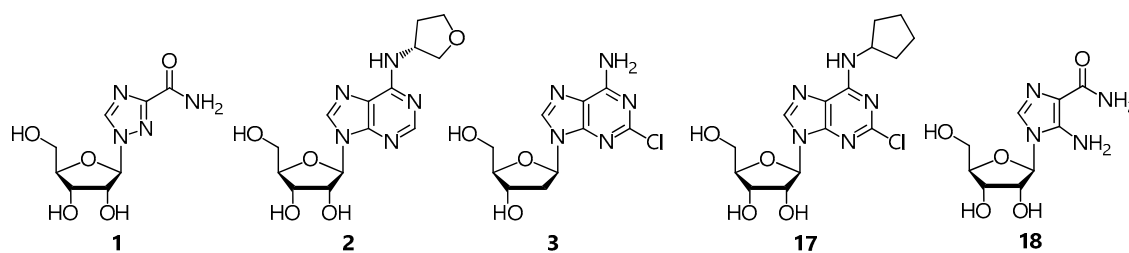

Figure 1. Ribavirin (**1**), Tecadenoson (**2**), Cladribine (**3**), 2-chloro-N^6-cyclopentyladenosine or CCPA (**17**), and Acadesine (**18**).

The synthesis of Ribavirin (**1**) has been achieved by both chemical and enzymatic approaches. The established glycosylation route involves the reaction of peracetylated β-D-ribofuranose with methyl 1,2,4-triazole-3-carboxylate, followed by aminolysis. Despite the good yields (54–83%) as well as the high regio- and stereoselectivity, high temperatures (135–170 °C) and high vacuum (15–55 mmHg) are required for the formation of the glycosydic bond [19,20]. Interestingly, when the reaction was carried out under slightly milder conditions (MW irradiation, 130 °C, 5 min and direct MPLC purification), the overall yield of Ribavirin was only 35% [21].

The synthesis of Ribavirin (**1**) by enzymatic transglycosylation was performed both by using whole cells (e.g., *Escherichia coli*, *A. hydrophila*, *Enterobacter aerogenes*, *Enterobacter gergoviae*) and isolated PNPs [22–33]. Either natural nucleosides or the suitable sugar phosphates were used as the ribose donor, resulting in variable yields (from 19% to 84%).

To date, the only synthetic strategy to obtain Tecadenoson (**2**) consists of nucleophilic substitution on either 2′,3′,5′-tri-*O*-acetyl-6-chloroinosine or 6-chloroinosine with (*R*)-3-aminotetrahydrofurane or its salts followed, when necessary, by deprotection with ammonia (reported yield: 68%) [34–36].

The direct glycosylation of a proper purine base (typically 2,6-dichloropurine) with a protected 1-chloro or 1-acetate ribose is the key step in numerous chemical syntheses of Cladribine (**3**) which can be obtained in variable yields ranging from 24% to 61% [37–39]. More elaborate multistep chemical strategies were also reported: they are based on either the 2′-deoxygenation of preformed protected adenosines [40,41], or the substitution with ammonia sources of purine nucleoside intermediates activated in 6-position [42–46].

Also, for Cladribine, enzymatic glycosylation (i.e., enzyme-catalyzed formation of the glycosydic bond) has been suggested as an alternative to the chemical route. Some examples of transglycosylation based on the use of PNPs (from *E. coli* and *Geobacillus thermoglucosidasius*) in mono- or bi-enzymatic processes have been reported [47,48].

While this research was ongoing, an *E. coli* PNP-catalyzed transglycosylation for the preparation of 2′-deoxynucleosides, including Cladribine, was developed by Mikhailov and co-workers who exploited a 7-methyl purine nucleoside iodide as the sugar donor [49].

The aim of this work was to enzymatically prepare Ribavirin, Tecadenoson, Cladribine, and some congeners (2-chloro-N^6-cyclopentyladenosine or CCPA, **17**, and Acadesine or AICAR, **18**, see Figure 1), by exploiting the well-established relaxed substrate specificity of *Ah*PNP [4–8] through a "one-pot, one-enzyme" transglycosylation based on the use of 7-methylguanosine (**7**) or 7-methyl-2′-deoxyguanosine iodide (**8**) as the sugar donor. It is worth noting that this route represents the first enzymatic synthesis of Tecadenoson.

In this context, we explored the use of 7-methylguanine arabinoside iodide (**9**) (Scheme 1) as the sugar donor for the synthesis of arabinosyl purine analogues such as the antiviral drug Vidarabine, as an alternative scheme to both the conventional chemical synthesis and the bi-enzymatic transglycosylation reaction [6].

4 (X = OH, Y = H)　　**7** (X = OH, Y = H)
5 (X = Y = H)　　　　**8** (X = Y = H)
6 (X = H, Y = OH)　　**9** (X = H, Y = OH)
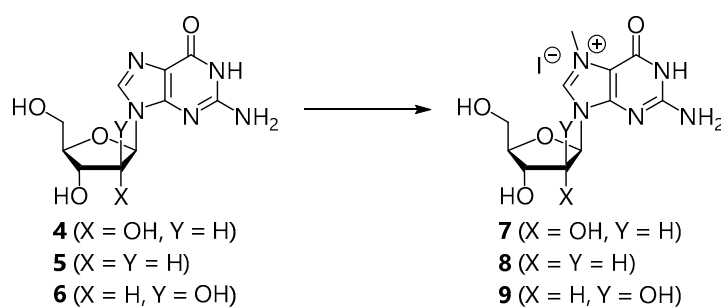
Scheme 1. Reagents and conditions (yield): CH$_3$I, DMF/DMSO (**7**: 87%; **9**: 89%) or CH$_3$I, DMSO, 20 °C (**8**: 80%).

2. Results and Discussion

2.1. Synthesis of the Sugar Donors

The three sugar donors, i.e., 7-methylguanosine iodide (**7**), 7-methyl-2′-deoxyguanosine iodide (**8**), and 7-methylguanine arabinoside iodide (**9**), were prepared by methylation of the corresponding nucleosides (Scheme 1).

The choice of CH$_3$I as the methylating agent and of the proper solvent (DMSO or a DMF/DMSO mixture) led to the selective formation of the iodide salts at N-7 (Scheme 1). Light exposure during the reaction at room temperature was avoided to prevent the decomposition of the final products, which had to be stored at −20 °C until use. The stability of 7-methylated nucleosides in DMSO or DMSO/DMF mixtures was found to depend on the nature of the sugar moiety, and posed a serious issue in the synthesis of 7-methyl-2′-deoxyguanosine iodide (**8**), as highlighted by ^{13}C NMR spectra

registered in DMSO-d_6 (see Supplementary Materials, Figure S1). The methylation reaction was thus performed by modifying a previously reported protocol [50] under controlled temperature (20 °C), short reaction time, and an excess of CH$_3$I in order to avoid any decomposition of **8** in DMSO. Higher temperatures (25–30 °C) and longer reaction times led to product decomposition.

No synthesis of 7-methylguanine arabinoside iodide (**9**) has been reported in the literature to date; thus, the same strategy (CH$_3$I in a DMSO/DMF mixture) was successfully applied to the methylation of arabinosylguanine (**6**), thus affording **9** in 83% yield. In contrast to **8**, 7-methylguanine arabinoside iodide (**9**) was as stable as the corresponding ribo-derivative (**7**). No decomposition products were detected both in DMSO/DMF and under bioconversion conditions (see below).

2.2. Synthesis of the Base Acceptors

The synthesis of 2-chloro-6-aminopurine (**11**), the base acceptor to prepare Cladribine (**3**), was achieved by treatment of 2,6-dichloropurine (**10**) with NH$_3$/MeOH under MW irradiation. Following the same approach, the base acceptors of Tecadenoson (**2**) and its congener, i.e., CCPA, were synthesized starting from 2,6-dichloropurine (**10**) or 6-chloropurine (**13**) and (*R*)-3-aminotetrahydrofuran hydrochloride/LiOH/EtOH or cyclopentylamine/*n*-BuOH, respectively (Scheme 2). Products were purified either by precipitation or by flash column chromatography in 35–69% yield. 1,2,4-Triazole-3-carboxamide (**15**) and 5-amino-1*H*-imidazole-4-carboxamide (**16**) (see Scheme 3) were commercially available.
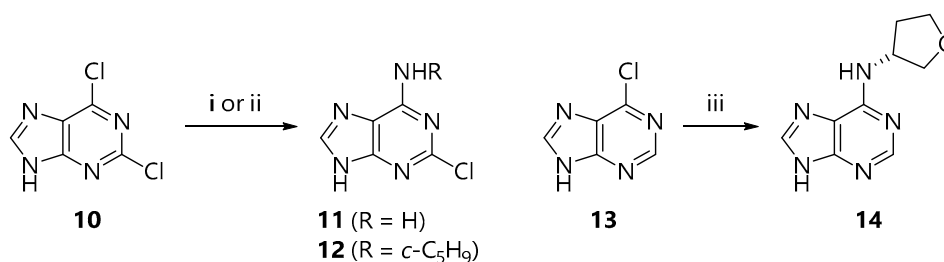
Scheme 2. Synthesis of adenine acceptors (i: NH$_3$, MeOH, 110 °C, MW, 66%; ii: *c*-C$_5$H$_9$NH$_2$, *n*-BuOH, reflux, 69%; iii: (*R*)-3-aminotetrahydrofuran hydrochloride, LiOH, EtOH, reflux, 35%).
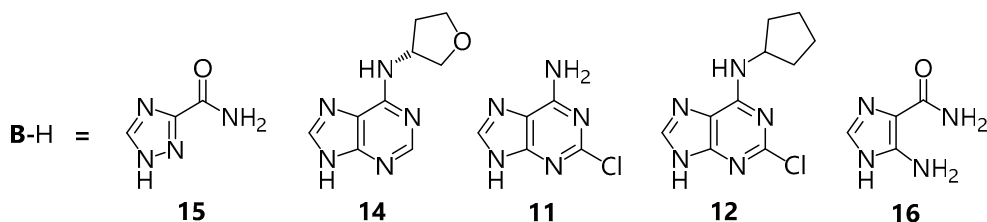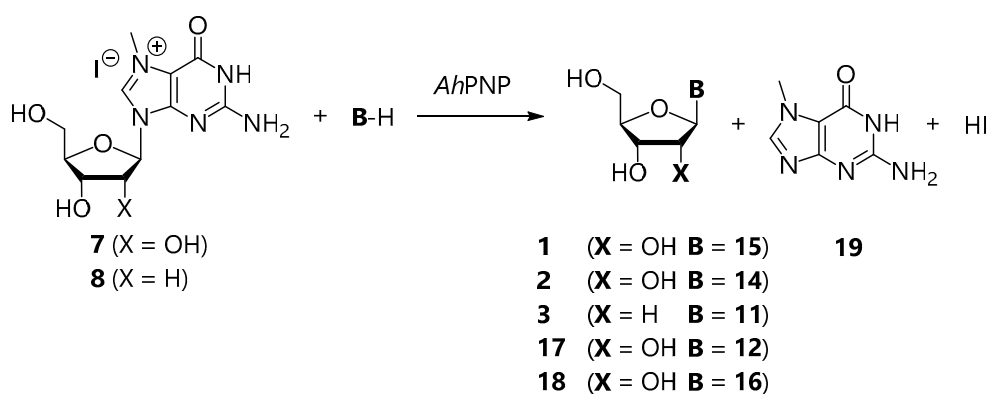
Scheme 3. Synthesis of nucleoside analogues **1–3** and **17–18** by enzymatic transglycosylation.

2.3. "One-Pot, One-Enzyme" Transglycosylations

"One-pot one-enzyme" transglycosylations were carried out starting from either 7-methylguanosine iodide (**7**) or 7-methyl-2′-deoxyguanosine iodide (**8**) as the sugar donors for the synthesis of the APIs **1–2** and **3**, respectively (see Scheme 3). As previously reported for nucleoside **7** [4,7], also the phosphorolysis reaction of **8** was almost complete and irreversible. In fact, the conjugated nucleobase of these nucleosides (7-methylguanine, **19**, see Scheme 3) was not recognized by *Ah*PNP as a substrate, thus assisting the shift of the reaction equilibrium toward the product formation.

Transglycosylation reactions occur under very mild conditions, generally in phosphate buffer at room temperature. The typical drawback of enzymatic reactions is the need to conjugate the poor solubility of substrates in aqueous media with the stability of the biocatalysts in organic solvent. Starting from a reaction set-up established in our labs, glycerol was used as the co-solvent in order to improve the substrate solubility and to preserve the enzyme activity (glycerol is routinely used as a protein preservative). Only in the case of Cladribine (**3**), DMSO was added as a second co-solvent besides glycerol (1 mL, 5% of the total volume) to overcome the very poor solubility of 2-chloro-6-aminopurine (**11**). As previously reported [8], the use of DMSO is quite well tolerated by *Ah*PNP.

As for the solubility issue, 7-methyl purine nucleosides are highly water soluble and their use as the sugar donors is, indeed, a true advantage. On the other hand, the conjugated base of these nucleosides (**7–9**), i.e., 7-methylguanine (**19**), is poorly water soluble and easily separates out the reaction, thus giving a further contribution to drive the reaction equilibrium. No less important, a further strength-point of 7-methyl purine nucleosides as the sugar donor relies on their straightforward and high-yielding preparation (see Section 2.1).

All reactions were carried out in 50 mM phosphate buffer (pH 7.5) containing 20% of glycerol (*v/v*) at room temperature (Scheme 3). Bioconversions were performed at an analytical scale (1 mM substrate concentration) by using a 1:1 donor/acceptor ratio. Reactions were monitored both by measuring the depletion of the nucleobase acceptor as well as the formation of the new nucleoside (see Materials and Methods). Conversions (end-point: 24 h) are reported in Table 1. All the HPLC peaks were assigned on the basis of the pure reference compounds, either purchased or synthesized (see Materials and Methods).

Table 1. Synthesis of the target nucleosides **1–3** and **17–18** by enzymatic transglycosylation. [1]

X [2]	B-H [2]	Product	Conversion
OH	**15**	**1 (Ribavirin)**	67%
OH	**14**	**2 (Tecadenoson)**	49%
H	**11**	**3 (Cladribine)**	56% [3]
OH	**12**	**17 (CCPA)**	52% [3]
OH	**16**	**18 (Acadesine)**	n.d.

[1] Experimental conditions: 50 mM KH$_2$PO$_4$, pH 7.5, and glycerol (20%), (substrate) = 1 mM, 1:1 donor/acceptor ratio, r.t., *Ah*PNP (21.5 mg mL^{-1}; 39 IU mg^{-1}) = 1.15 IU or 80 IU; time monitoring (HPLC): 1, 3, 6, and 24 h, endpoint = 24 h. [2] X and B-H as in Scheme 3. [3] For the synthesis of **3** and **17**, DMSO (5% or 10% *v/v*, respectively) was used as the second co-solvent besides glycerol. n.d. = not detected.

As recalled in the Introduction, the synthesis of nucleoside-based APIs by an enzymatic transglycosylation reaction has been investigated by many authors (for a comprehensive review, see References [3,51]; for a recent example see Reference [52]). However, the enzymatic synthesis of Tecadenoson has been reported herein for the first time (see Supplementary Materials, Figure S7). Taking into account this result and the evidence that *Ah*PNP can accept a wide array of 6-substituted purines as substrates, we also successfully synthesized CCPA (**17**), the congener of Tecadenoson (Scheme 3). In this case, as the base acceptor (**12**) was not soluble in the buffer–glycerol mixture even in the presence of 5% DMSO (as applied in the synthesis of Cladribine), the biotransformation was carried out in a sort of "fed batch" mode. A 10 mM stock solution of **12** in DMSO was progressively

added to the reaction upon monitoring the rate of phosphorolysis and the transglycosylation reaction (see Supplementary Materials, Figure S5). The final percentage of DMSO was 10% *v/v*. It is worth reporting that a large excess of *Ah*PNP was used in this case, as the formation of the target nucleoside was hardly detectable when using 1.15 U of enzyme. This result prompted us to further extend this approach to the synthesis of other ribo-derivatives such as Acadesine (or AICAR, **18**), a congener of Ribavirin. This reaction was performed under standard conditions (glycerol–buffer) by using the same excess of *Ah*PNP as for CCPA. At this stage of the project, in fact, the goal was to assess whether the biocatalyst could synthesize Acadesine. Surprisingly, whereas in the case of Ribavirin a conversion of 67% was registered after 24 h, the formation of Acadesine was not observed (see Supplementary Materials, Figures S4 and S6).

As it is well known, the chemical route to purine 2′-deoxyribonucleosides is even more challenging than that to its ribo-counterparts [2]. Therefore, the availability of 7-methyl-2′-deoxyguanosine iodide would represent a valuable tool for an alternative synthetic approach, as proven by the bioconversion of Cladribine (see Table 1). However, this sugar donor was found to be less stable than 7-methylguanosine iodide in aqueous medium, in agreement with a very recent report by Mikhailov et al. [49]. We have found that this molecule is unstable also in DMSO at room temperature, as indicated from the appearance of extra signals in the ^{13}C NMR spectrum recorded at two-hour intervals in DMSO, clearly showing that the decomposition of the nucleoside had occurred (see Supplementary Materials, Figure S1). Dimethyl sulfoxide was used in the synthesis of Cladribine (**3**) as the second co-solvent. The evidence of the poor stability of 7-methyl-2′-deoxyguanosine iodide both in buffer solutions and in DMSO makes this molecule a substrate which is difficult to handle in preparative applications.

As a natural continuation of this study, our efforts were then focused on the synthesis of arabinosyl purine nucleosides by using the newly prepared 7-methylguanine arabinoside iodide (**9**) as the sugar donor. Vidarabine (arabinosyladenine) was selected as the target API as its enzymatic synthesis, although through a bi-enzymatic approach [6,9], it was successfully achieved even at a preparative scale with a good yield and purity (3.5 g/L, 53% yield, 98.7% purity). Surprisingly, when using 7-methylguanine arabinoside iodide (**9**), neither phosphorolysis, nor transglycosylation, thereof, were observed. This result suggests the need for a deeper understanding of the structural requisites for the enzyme-substrate molecular recognition; this is a necessary step to rationalize all the data collected over the years about the substrate specificity of *Ah*PNP as well as to further widen the exploitation of this enzyme in the bio-catalyzed synthesis of modified nucleosides, also at a preparative scale. In this regard, taking into account that the synthesis of Ribavirin (**1**), Tecadenoson (**2**), and Cladribine (**3**) were performed under screening conditions by using a 1:1 donor/acceptor ratio, conversions values were remarkable (≥50%) and foresee considerable room for improvement.

3. Materials and Methods

3.1. General

3.1.1. Chemicals

Solvents and reagents were purchased from Sigma–Aldrich (Milano, Italy), Fluorochem (Hadfield, Derbyshire, UK), Fluka (Milwaukee, WI, USA), Merck (Darmstadt, Germany), and were used without any further purification, unless stated otherwise. Dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), acetone, methanol (MeOH), and ethanol (EtOH) were distilled before use. All other solvents were of HPLC grade.

Purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNP) was provided by Gnosis S.p.A. (Desio, MB, Italy). Specific activity toward inosine was 39 IU·mg^{-1} (stock solution 21.5 mg·mL^{-1}) [4]. One IU corresponded with an amount of enzyme that converts one mmol of inosine into hypoxanthine per min.

3.1.2. Methods

Analytical TLC was performed on silica-gel F254 precoated aluminum sheets (0.2 mm layers, Merck, Darmstadt, Germany). Elution solvent: CH_2Cl_2–MeOH, 9:1. Detection: UV lamp (λ 254 nm) and 4.5% *w/v* $CeSO_4$/$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution or 5% *w/v* ninhydrin solution in EtOH followed by heating at 150 °C.

Flash column chromatography was performed using silica gel 60, 40–63 μm (Merck, Darmstadt, Germany). Reaction performed by microwave (MW) irradiation (300 W) were run in a Biotage Initiator + apparatus (Biotage, Uppsala, Denmark).

^{1}H and ^{13}C spectra were recorded at 400.13 and 100.61 Hz, respectively, on a Bruker AVANCE 400 spectrometer equipped with a TOPSPIN software package (Bruker, Karlsruhe, Germany) at 300 K, unless stated otherwise. ^{1}H and ^{13}C chemical shifts (δ) are given in parts per million and were referenced to the solvent signals (δ_H 3.31–δ_C 49.00, δ_H 2.50–δ_C 39.52 ppm from tetramethylsilane (TMS) for CD_3OD and DMSO-d_6, respectively). The ^{13}C NMR signal multiplicities were based on APT (attached proton test) spectra. The ^{13}C NMR signals were assigned with the aid of ^{1}H-^{13}C correlation experiments (heteronuclear multiple quantum correlation spectroscopy, HMQC, and heteronuclear multiple bond correlation spectroscopy, HMBC).

Electrospray ionization mass spectra (ESI-MS) were recorded on a ThermoFinnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK).

The pH measurements were performed by using a 718 Stat Titrino pHmeter from Metrohm (Herisau, Switzerland).

Enzymatic reactions were monitored by HPLC using a Merck Hitachi L-7000 La-Chrom liquid chromatographer equipped with a UV-Vis detector, an autosampler (injection volume: 20 μL), and a column oven (instrument 1), or a Chromaster 600 bar system, Merck Hitachi VWR equipped with a UV-Vis detector, an autosampler (injection volume: 20 μL), and a column oven (instrument 2).

Chromatographic conditions: column, Phenomenex Gemini C_{18} (5 μm, 250 × 4.6 mm, Supelco) or SepaChrom C_{18}-Extreme (5 μm, 250 × 4.6 mm); flow rate: 1.0 mL·min^{-1}; λ: 260 nm (225 nm for Ribavirin synthesis); temperature: 35 °C; eluent: 50 mM K_2HPO_4 buffer pH 4.5 (A) and MeOH (B); method: from 3% to 65% B (20 min), 65% B (5 min), from 65% to 3% B (0.1 min), 3% B (15 min). Under these conditions the following retention times (t_R) were registered: 7-methylguanosine iodide (**7**) (6.64 min); 7-methyl-2'-deoxyguanosine iodide (**8**) (7.20 min); Ribavirin (**1**) (4.53 min); 1,2,4-triazole-3-carboxamide (**15**) (3.25 min); Tecadenoson (**2**) (19.32 min); 6-(3-aminotetrahydrofuranyl)purine (**14**) (17.46 min); Cladribine (**3**) (18.62 min); 2-chloro-6-aminopurine (**11**) (15.16 min); CCPA (**17**) (26.39 min); 2-chloro-N^6-cyclopentyladenine (**12**) (27.60 min); Acadesine or AICAR (**18**) (6.47 min); 5-amino-1*H*-imidazole-4-carboxamide (**16**) (3.72 min); 7-methylguanine (**19**) (7.97–8.06 min). Retention times of CCPA (**17**) and 7-methylguanine (**19**) were assigned by exclusion, upon analyzing the profile of each chromatogram. The samples from the enzymatic reactions were analyzed after filtering off the enzyme through centrifugal filter devices (10 kDa MWCO, VWR International, Milano, Italy).

3.2. Chemical Synthesis of Sugar Donors

7-Methylguanosine iodide (**7**). The title compound was synthesized in 87% yield as previously reported [4]. 7-Methyl-2'-deoxyguanosine iodide (**8**). The title compound was prepared following a published procedure with some modifications [50]. Under inert atmosphere, a solution of **5** (160 mg, 0.60 mmol, 1.00 equivalent), and CH_3I (0.30 mL, 4.82 mmol, 8.03 equivalent) in dry DMSO (1.20 mL) was stirred at 20 °C for 4 h 30'. The mixture was continuously protected from light exposure, and as the solution slowly turned brown-red, the substrate disappearance was monitored by TLC (EtOH–H_2O, 4:1; R_f = 0.80). Cold $CHCl_3$ (15 mL) was added to precipitate a pale-yellow powder and the suspension was decanted at 0 °C for 2 h. The precipitate was filtered, washed with cold $CHCl_3$, and dried to get **8** as an off-white powder, which was stored at −20 °C (197 mg, 0.48 mmol, 80%). R_f: 0.21 (EtOH–H_2O, 4:1). HPLC t_R: 7.20 min. ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 11.67 (s, 1H, NH^1), 9.28 (s, 1H, H^8),

7.18 (br s, 2H, N^{2}H$_2$), 6.20 (t, *J* = 6.0 Hz, 1H, H$^{1'}$), 5.40 (br s, 1H, OH$^{3'}$), 4.98 (br s, 1H, OH$^{5'}$), 4.37 (dd, *J* = 9.1, 4.6 Hz, 1H, H$^{3'}$), 4.00 (s, 3H, N^{7}CH$_3$), 3.93 (q, *J* = 3.9 Hz, 1H, H$^{4'}$), 3.62 (dd, *J* = 12.1, 4.2 Hz, 1H, H$^{5'a}$, partially overlapped with H$^{5'b}$), 3.57 (dd, *J* = 12.1, 4.2 Hz, 1H, H$^{5'b}$, partially overlapped with H$^{5'a}$), 2.55–2.47 (m, 1H, H$^{2'a}$, partially covered by DMSO), 2.40 (ddd, *J* = 13.4, 6.2, 4.8 Hz, 1H, H$^{2'b}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ (ppm) 156.0 (C^2), 153.9 (C^6), 149.3 (C^4), 136.7 (C^8), 108.1 (C^5), 89.1 (C$^{4'}$), 85.8 (C$^{1'}$), 70.2 (C$^{3'}$), 61.3 (C$^{5'}$), 40.5 (C$^{2'}$, partially covered by DMSO), 36.1 (N^{7}CH$_3$). MS (ESI$^+$): *m/z* calcd. for [C$_{11}$H$_{16}$N$_5$O$_4$]$^+$: 282.12; found: 166.3 [M-2'-deoxyribosyl]$^+$, 282.1 [M]$^+$, 305.3 [M + Na]$^+$, 563.7 [2M]$^+$. MS (ESI$^-$): *m/z* calcd. for [I]$^-$: 126.91; found: 127.2 [M]$^-$.

7-Methylguanine arabinoside iodide (**9**). Under inert atmosphere, a solution of **6** (100 mg, 0.35 mmol. 1.00 equivalent), synthesized as previously reported [53], and CH$_3$I (0.14 mL, 2.25 mmol, 6.43 mmol) in a dry DMF/DMSO mixture (3:1 *v/v*, 1.00 mL) was stirred at room temperature for 6 h. The mixture was continuously protected from light exposure, and as the solution slowly turned yellow, the substrate disappearance was monitored by TLC (EtOH–H$_2$O, 7:3; R_f = 0.83). The mixture was diluted with H$_2$O (50 mL) and freeze-dried until complete removal of DMSO. The resulting pale-yellow crude was suspended in dry acetone (2.5 mL), filtered, washed with few cold dry acetone, and dried to get **9** as an off-white powder, which was stored at −20 °C (133 mg, 0.31 mmol, 89%). R_f: 0.53 (EtOH–H$_2$O, 7:3). ^{1}H NMR (DMSO-d_6, 400 MHz):δ (ppm) 11.68 (br s, 1H, NH^1), 9.22 (s, 1H, H^8), 7.21 (br s, 2H, N^{2}H$_2$), 6.17 (d, *J* = 4.3 Hz, 1H, H$^{1'}$), 5.82 (d, *J* = 5.3 Hz, 1H, OH$^{2'}$), 5.64 (d, *J* = 4.3 Hz, 1H, OH$^{3'}$), 5.04 (t, *J* = 5.4 Hz, 1H, OH$^{5'}$), 4.17 (dd, *J* = 8.6, 4.4 Hz, 1H, H$^{2'}$), 4.11 (dd, *J* = 7.5, 3.7 Hz, 1H, H$^{3'}$), 4.06 (s, 3H, N^{7}CH$_3$), 3.90 (dd, *J* = 8.9, 5.1 Hz, 1H, H$^{4'}$), 3.70 (dd, *J* = 11.5, 5.0 Hz, 1H, H$^{5'a}$, partially overlapped with H$^{5'b}$), 3.64 (dd, *J* = 11.6, 5.4 Hz, 1H, H$^{5'b}$, partially overlapped with H$^{5'a}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ (ppm) 156.2 (C^2), 153.8 (C^6), 149.6 (C^4), 137.8 (C^8), 107.6 (C^5), 86.5 (C$^{4'}$), 86.0 (C$^{1'}$), 75.5 (C$^{2'}$), 75.4 (C$^{3'}$), 61.3 (C$^{5'}$), 36.1 (N^{7}CH$_3$). MS (ESI$^+$): *m/z* calcd. for [C$_{11}$H$_{16}$N$_5$O$_5$]$^+$: 298.11; found: 166.2 [M-arabinosyl]$^+$, 297.1 [M − H]$^+$, 298.0 [M]$^+$. MS (ESI$^-$): *m/z* calcd. for [I]$^-$: 126.91; found: 127.1 [M]$^-$.

3.3. Chemical Synthesis of Base Acceptors and Tecadenoson

2-Chloro-6-aminopurine (**11**). The title compound was prepared following a published procedure with some modifications [54]. Under inert atmosphere, a solution of **10** (113 mg, 0.60 mmol, 1.00 equivalent) in a 30% aq. NH$_3$–MeOH mixture (2:3 *v/v*, 11.3 mL) was stirred under MW irradiation at 110 °C for 14 h. The solution was evaporated and the resulting light-blue solid was suspended in H$_2$O. The precipitate was filtered and dried to get **11** as a white powder (67 mg, 0.40 mmol, 66%).^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 8.13 (s, 1H, H^2), 7.62 (s, 2H, NH$_2$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ (ppm) 156.1, 153.8, 140.8. MS (ESI$^+$): *m/z* calcd. for [C$_5$H$_4$ClN$_5$]$^+$: 169.02; found: 507.31 [3M]$^+$. MS (ESI$^-$): *m/z* calcd. for [C$_5$H$_4$ClN$_5$]$^-$: 169.02; found: 168.13 [M − H]$^-$.

2-Chloro-N^6-cyclopentyladenine (**12**). The title compound was prepared following a published procedure with some modifications [55]. Under inert atmosphere, a suspension of **10** (142 mg, 0.75 mmol, 1.00 equivalent), cyclopentylamine (0.22 mL, 2.25 mmol, 3.00 equiv) in dry *n*-BuOH (1.50 mL) was refluxed for 4 h. The solvent was evaporated and the resulting crude was purified by flash chromatography (CH$_2$Cl$_2$–MeOH, 9.7:0.3) to get **12** as a white powder (191 mg, 0.52 mmol, 69%). R_f: 0.33 (CH$_2$Cl$_2$–MeOH, 9.7:0.3). ^{1}H NMR (CD$_3$OD, 400 MHz): δ (ppm) 8.04 (s, 1H, H^8), 4.56 (br s, 1H, H^a), 2.33–2.05 (m, 2H, H^b), 1.83 (qd, *J* = 10.5, 8.8, 5.0 Hz, 2H, H^b), 1.78–1.68 (m, 2H, H^c), 1.63 (ddt, *J* = 14.0, 8.1, 5.2 Hz, 2H, H^c). ^{13}C NMR (CD$_3$OD, 100 MHz): δ (ppm) 153.5, 139.6, 112.2, 52.0, 32.4, 23.9. MS (ESI$^+$): *m/z* calcd. for [C$_{10}$H$_{12}$ClN$_5$]$^+$: 237.08; found: 259.99 [M + Na]$^+$.

6-(3-Aminotetrahydrofuranyl)purine (**14**). Under inert atmosphere, a suspension of **13** (154 mg, 1.00 mmol, 1.00 equivalent), (*R*)-3-aminotetrahydrofuran hydrochloride (595 mg, 6.00 mmol, 6.00 equivalent) and LiOH (250 mg, 6.00 mmol, 6.00 equivalent) in EtOH (3.5 mL) was refluxed for 6 h. The solution was evaporated and the residue was purified by flash chromatography (CH$_2$Cl$_2$–MeOH, 9.3:0.7) to get **14** as a white powder (72 mg, 0.35 mmol, 35%). ^{1}H NMR (CD$_3$OD, 400 MHz): δ (ppm) 8.28 (s, 1H, H^2), 8.10 (s, 1H, H^8), 4.92–4.79 (m, 1H, H^a), 4.16–3.99 (m, 2H, H^b, H^c), 3.91 (td, *J* = 8.4, 5.5 Hz,

1H, H^c), 3.81 (dd, *J* = 9.2, 3.4 Hz, 1H, H^b), 2.41 (ddt, *J* = 13.0, 8.2, 7.2 Hz, 1H, H^d), 2.04 (dddd, *J* = 13.0, 7.6, 5.4, 3.7 Hz, 1H, H^d). ^{13}C NMR (CD$_3$OD, 100 MHz): δ (ppm) 152.3, 73.0, 66.7, 51.4, 32.4. MS (ESI$^+$): *m/z* calcd. for [C$_9$H$_{11}$N$_5$O]$^+$: 205.10; found: 206.05 [M + H]$^+$, 228.06 [2M + Na]$^+$.

Tecadenoson (**2**). The title compound was prepared following a published procedure with some modifications [35]. Under inert atmosphere, a suspension of 6-chloroinosine (143 mg, 0.50 mmol, 1.00 equivalent), (*R*)-3-aminotetrahydrofuran hydrochloride (148 mg, 1.50 mmol, 3.00 equivalent) and triethylamine (0.21 mL, 1.50 mmol, 3.00 equivalent) in EtOH (4.0 mL) was refluxed for 4 h. The solution was evaporated and the residue was purified by flash chromatography (CH$_2$Cl$_2$–MeOH, 9:1) to get **2** as a white powder (93 mg, 0.28 mmol, 55%). ^{1}H NMR (DMSO-*d$_6$*, 400 MHz): δ (ppm) 8.39 (s, 1H, H^2), 8.24 (s, 1H, H^8), 8.14–7.93 (m, 1H, N^{6}H), 5.90 (d, *J* = 6.1 Hz, 1H, H$^{1'}$), 5.45 (s, 1H, OH$^{2'}$), 5.37 (t, *J* = 5.6 Hz, 1H, OH$^{5'}$), 5.19 (s, 1H, OH$^{3'}$), 4.72 (s, 1H, NH^6), 4.61 (t, *J* = 5.5 Hz, 1H, H$^{2'}$), 4.15 (s, 1H, H$^{3'}$), 4.10 (q, *J* = 5.2 Hz, 3H, H^a, H^b, H^c), 4.01–3.83 (m, 1H, H$^{3'}$), 3.84–3.43 (m, 3H, H$^{4'}$, H^b, H^c), 2.20 (dq, *J* = 14.6, 7.6 Hz, 1H, H^d), 2.04 (br, 1H, H^d). ^{13}C NMR (DMSO-*d$_6$*, 100 MHz): δ (ppm) 153.2, 151.9, 87.5, 85.9, 72.4, 71.0, 66.5. MS (ESI$^+$): *m/z* calcd for [C$_{14}$H$_{19}$N$_5$O$_5$]$^+$: 337.14; found: 360.04 [M + Na]$^+$.

3.4. Enzymatic Synthesis of Nucleoside Analogues: General Procedure of Transglycosylation Reactions

Purine nucleoside phosphorylase from *A. hydrophila* (1.15 or 80 IU) was added to a solution of 50 mM KH$_2$PO$_4$ buffer pH 7.5 and 20% (*v/v*) glycerol containing **7** or **8** (1 mM) and the modified nucleobase **B**-H (1 mM, see Table 1). In the case of Cladribine (**3**) and CCPA (**17**), DMSO was added as the second co-solvent (5% or 10% *v/v*, respectively). The final reaction volume was 20 mL or 5 mL. The mixture was gently stirred (rolling shaker) at room temperature. Aliquots (200 μL) of the reaction mixture were withdrawn at fixed times (1, 3, 6, 24 h), and filtered by centrifugation (MWCO 10 kDa, 5 min, 12,000 rpm). The supernatant was diluted 1:4 with the mobile phase and analyzed by HPLC (injection volume: 20 μL). Conversions were estimated by Equations (1) and (2):

$$\text{Base consumption (\%)} = \frac{\text{base area}}{\text{base area } + \text{ nucleoside area}} \times 100 \tag{1}$$

$$\text{Nucleoside formation (\%)} = \frac{\text{nucleoside area}}{\text{base area } + \text{ nucleoside area}} \times 100 \tag{2}$$

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/9/4/355/s1, Figure S1: ^{13}C-NMR monitoring of 7-methyl-2'-deoxyguanosine iodide (**8**) stability, Figure S2: ^{1}H-NMR and ^{13}C-NMR spectra of 7-methylguanine arabinoside iodide (**9**), Figure S3: ^{1}H-NMR spectrum of Tecadenoson (**2**), Figure S4: HPLC monitoring of the enzymatic synthesis of Ribavirin (**1**), Figure S5: HPLC monitoring of the enzymatic synthesis of CCPA (**17**), Figure S6: HPLC monitoring of the enzymatic synthesis of Acadesine (**18**), Figure S7: HPLC monitoring of the enzymatic synthesis of Tecadenoson (**2**).

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